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PCR-RFLP of *Coxiella burnetii* Plasmids Isolated from Raw Milk Samples in Iran

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ABSTRACT

Background and Aim: Several methods have been employed to identify *Coxiella burnetii* isolates based on the specific *C. burnetii QpH1* plasmid to distinguish the acute form from the chronic form of Q fever disease in humans and animals owing to the presence of unique gene sequences in this plasmid. Therefore, the present study aimed to investigate the panel of nucleic acid fragments resulting from the enzymatic cleavage in the *QpH1* plasmid isolated from cow and buffalo milk by nested polymerase chain reaction (Nested-PCR).

Materials and Methods: A total of 86 isolates of *C. burnetii QpH1* plasmid, which was confirmed by the Nested-PCR method in 2018, were used to determine the RFLP panel of the *QpH1* plasmid. Plasmids were first extracted with the kit and were then affected by the *Hph1* restriction enzyme. Additionally, 4 nucleic acid samples were sent to *Pishgam* Company for sequencing with the *IS1111* gene primer.

Results: Based on the results of the PCR-RFLP test, all plasmid samples showed a similar two-fragment pattern under the influence of *Hph1*. The results of the nucleic acid sequencing of all 4 samples indicated that they had a *C. burnetii* type (Nine Mile RSA493 strain).

Conclusion: RFLP patterns exhibited no difference on the *C. burnetii QpH1* plasmid isolated from cow and buffalo milk. Hence, all isolates were genetically identical, and the infection in animals could originate from one *C. burnetii* strain (Nine Mile RSA493 strain).

Keywords: Buffalo, Cattle, *Coxiell burnetii,* Plasmid, PCR-RFLP

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1. Introduction

One of the obligate intracellular bacteria that induce severe Q fever and chronic endocarditis in humans is *Coxiella burnetii*. The fever is similar to flu and self-limiting; it can be treated easily using antibiotics, provided the diagnosis is carried out properly. The disease needs antibiotic therapy for a long time as the infection may lead to endocarditis or granulomatous hepatitis. It is imperative to differentiate *C. burnetii* rapidly in clinical specimens. The reason for this is the fact that proper antibiotic therapy can result in a better prognosis. The standard diagnosis of Q fever includes serological tests, given that isolating *C. burnetii* from patients is a long, complicated, and risky process. Still, serological tests are ineffective for treating afflicted patients as they only give a retrospective diagnosis **(1, 2)**.

Several genotyping methods have been developed to distinguish *C. burnetii* isolates. One of these techniques is restriction enzyme fragment length polymorphism (RFLP) analysis of genomic DNA and PCR-RFLP of specific genes **(1)**. Importance of RFLP Technology; Several studies have shown the high genetic diversity of *C. burnetii* strains by the RFLP method. In a study by Jaguar *et al.* The relationship between the geographical distributions of *C. burnetii* is shown by the RFLP method. An association between the RFLP group and the pathogenicity of the *C. burnetii* strain in a rodent acute Q fever model was demonstrated **(2)**.

Restriction fragment length polymorphisms (RFLP) in chromosomal have been explored in recent literature. Further research demonstrates the study of RFLP patterns using sodium dodecyl sulfate-polyacrylamide **(3)** or pulsed-field gel electrophoresis **(4)**. Accordingly, making it possible to establish the creation of six distinct genomic groups (I through VI) is an important implication of the subsequent research. While the groups IV and V contained isolates from chronic disease patients, isolates from acute disease patients can be categorized into genomic groups. Moreover, presenting the categorized isolates in genomic groups I, II, and III and group IV is just to contain plasmid *QpH1* and plasmid *QpRS*, respectively. Also, group V isolates are plasmid-less. However, *QpRS*-homologous sequences are marked in chromosomal genome sequences of the group V isolates **(5, 6)**. Although recent investigations indicate that the group VI isolates might include *QpH1* and *QpDG,* which are demonstrated to be virulent in guinea pigs, these isolates were suggested to be contained the plasmid *QpDG*. Further investigations have been carried out to specify a variety of isolates utilizing RFLP/ pulsed-field gel electrophoresis with various restriction enzymes **(7)**, multispacer sequence typing **(8)**, multiple loci variable number of tandem repeats analysis **(9, 10)**, and microarrays **(11)**. Those are set to discriminate the isolates into 36 distinct genotypes.

Identification of the *C. burnetii* plasmid can provide some basic information in the differential diagnosis and epidemiological investigation of Q fever. However, it was not possible to show a correlation between the six genomic groups of *C. burnetii* and their pathogenicity or clinical manifestations **(12)**.

After completing the nine-mile phase I (9Mi / I) genomic sequence, 20 unique copies of transposed *IS1111* were identified. Previous studies of insertion sequences have shown that the transposase coding region is surrounded by two sets of terminal reverse repeats, formerly known as medial and lateral reverse repeats **(13)**. Inverse internal repeats are supposed to indicate the IS element term. *IS1111* is expected to form a circular intermediate, bringing reverse internal repeats closer together to form a strong promoter, which in turn increases transposase expression. The outer reverse repeat is thought to be a chromosomal sequence that forms a stem-loop structure that serves as a recognition site for the insertion of the IS element but is not part of the IS element itself. There are more than 50 copies of 9 Mi/I genome in such chromosomal target sequence **(14)**.

Furthermore, the fragment of a transposon-like repetitive element to is utilized detect the bacterium in clinical samples, such as placental bits, genital and fecal swabs, urine, liver, spleen, placenta, heart valves, milk, blood, and serum samples **[**15**,** 16**]**. The analytical sensitivity of the Trans-PCR was found to be 10^9 (sometimes even 10 −1) *C. burnetii* particles per reaction mixture.

Due to the characterization of these plasmids, *C. burnetii* was classified into 6 genomic groups. The QpH1 plasmid was first obtained from tick isolates and was also detected in most isolates derived from domestic mites. Currently, the following new genotyping techniques are being developed. B. Multiple locus variable number tandem repeat analysis (VNTR) (MLVA), multi-spacer sequence typing (MST), and single nucleotide polymorphism (SNP) genotyping. The RFLP method is better than the sequence-based method because epidemiological studies should identify the stage of the disease (acute or chronic infection) and the genotype of the bacteria to determine how to treat the infection. It's cheap and easy **(15)**. This report describes the PCR-RFLP assay results for directly identifying the *C. burnetii* plasmid *QpH1* gene in dairy and buffalo.

2. Materials and Methods

Milk Sample

In the present research, 86 milk samples for the *QpH1* gene that were positive by nested PCR in our previous study were selected. The four DNA samples of *C. burnetii* based on *the IS1111* gene **(16)** were sequenced to confirm.

DNA Extraction

DNA extraction of the selected Milk samples was carried out according to the kit's manufacturer instructions (Favorgen, Taiwan). The quality and amount of extracted DNA were evaluated by NanoDrop 2000c (Thermo Scientific, USA). Extracted DNA samples were kept at -20°C until use in PCR.

Positive Control

The *C. burnetii* isolates used in this study comprised one *QpH1* plasmid-containing strain (*C. burnetii* standard Nine Mile strain RSA 493).

Table 1. Primer sequences for detection of *QpH1* gene by nested-PCR.

Protocol	Primer Name	Sequence 5'----3'	PCR product size (bp)	PCR condition (Cycle)	Reference
PCR	CB5	ATAATGAGATTAGAACAACCAAGA	977	94 for 4m, 94 for2m, 53 for1m, 72 for2m, 72/5. (35)	(17)
	CB6	TCTTTCTTGTTCATTTTCTGAGTC			
nested-PCR	QpH1-F	CTCGCTGACGGAAGAGGATCTTTT	602	94 for3m, 94 for45s, 50 for45s, 72 for45s, 72 for5m. (35)	This study
	QpH1-R	TAACACTGCCCGTCGCTTTACT			

Nested-PCR

In order to the molecular detection of the *QpH1* plasmid, nested PCR was applied. The first stage pares primers for the detection of the *QpH1* gene used based on what Zhang *et al.* previously described **(17)**. For the nested- PCR, designed the second set of primers for QpH1-F and QpH1R using AmplifX (Version 2.2.0) software (table 1).

RFLP of QpH1 Plasmid

In order to consider the genetic map of QPH1 gene, restriction enzyme Hph1 (Thermo Fisher Scientific Inc. USA) (Takapouzist Co., Iran) was employed. The recommended protocol for the digestion of PCR products directly after amplification is based on enzyme *HphI*. Incubate at 37°C for 1-16 hours that explaind in (Table 2).

Table 2. *HphI* enzyme protocol.

name	Volume (µL
PCR reaction mixture	10
Nuclease-free water	18
10X Buffer B	2
HphI	1-2

Detection of PCR Products

The amplified products of PCR and the restricted products were examined by electrophoresis in a 2.5% agarose gel, stained with a safe stain (0.5 mg/mL), visualized under UV illumination (TM-20; UVP, Inc.) at 320 nm, and photographed.

3. Results

The specificity of the nested PCR with primers QpH1-F, QpH2-R was also confirmed by digesting the amplified products with *Hph1*, which yielded 168-bp and 279-bp fragments (Figure 1). All studied specimens contained two pieces of 168 bp and 279 bp. The PCR-RFLP results for *QpH1* showed that the enzymatic cleavage of the positive PCR product caused no significant difference between patterns of enzymatic cleavage. In addition to confirming the PCR result, PCR-RFLP indicated that *C. burnetii* found in all specimens belonged to a single strain called *C. burnetii* Nine Mile.

Confirmation of *C. burnetii* Based on *IS1111* Gene and Phylogenetic Analysis

The results confirmed the presence of *C. burnetii*. After modifying and aligning the sequences in MEGA-10, it was shown that all four sequences were completely similar and contained 160 bp. The Basic Local Alignment Search Tool (BLAST) results also revealed 100% similarity of these sequences with more than 50 sequences in the gene bank from different sources. Therefore, considering the 100% similarity with other isolates in different sources and regions, there was no need to plot a phylogenetic tree. Reference No. of these four sequences in the gene bank is as follows: MW172976, MW172975, MW172974, MW172973.

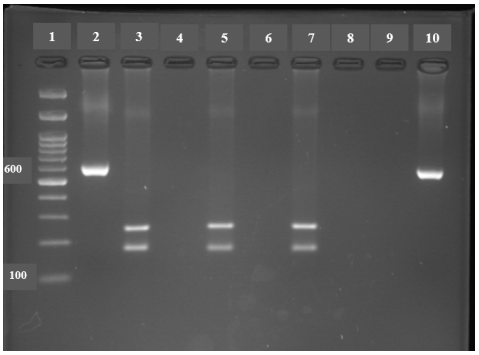

Figure 1. Specificity of the nested PCR with primers CB5- CB6 and QpH1-F-QpH1-R demonstrated the 602-bp amplification products were digested with *Hph1*, electrophoresed on agarose gel, and stained with safe stain.

4. Discussion

Although *C. burnetii* is considered homologous by serological methods and sequence analysis of the 16S rRNA gene, the data presented in this study highlights no genetic diversity among isolates. RFLP analysis by GE should be seen as a powerful tool for isolation typing **(18)**.

Q fever is a zoonotic disease that is found in cattle and buffaloes in northwestern Iran **(16, 19-24)**. Environmental pollution in infected farms is due to the excretion of bacteria by infected animals through the placenta, fetal fluids, vaginal secretions, feces,

urine, and milk. In animals, the prevalence of Q fever in females is higher than in males due to the greater susceptibility of females to males. Pregnancy is also an important factor in the development of Q fever, and a higher incidence of the disease has been reported in cattle during pregnancy **(15)**.

Q fever is not well known in Iran. Lack of knowledge about this disease leads to undiagnosed and under-reported cases of Q fever. *C. burnetii* is characterized by very high infectivity. *C. burnetii* can cause acute and chronic forms in humans and is isolated from a wide range of warm-blooded animals and arthropods. Previous studies have identified several forms of plasmids in this bacterium **(25, 26)**.

So far, The *C. burnetii* isolate classification was introduced primarily to determine the pathogenicity of new isolates. Understanding the relationships between *C. burnetii* isolates was of little interest, probably because the differentiation method did not reveal sufficient diversity and a relatively small number of isolates were analyzed. The mentioned difference in RFLP between North American and European *C. burnetii* isolates shows a similar restriction pattern. Until today, Diversity between *C. burnetii* isolates has never been used to quantify associations **(27, 28)**.

Rijks et al., working on the molecular epidemiology of *C. burnetii* in ruminants in the Netherlands, concluded that the genetic background of *C. burnetii* in ruminants was responsible for the prevalence of Q fever in humans **(18)**. Astobiza *et al.* also carried out a study in 2012 on the genotype of *C. burnetii* isolated from domestic ruminants in northern Spain and found that there is a significant genetic diversity of *C. burnetii* **(29)**.

The QpH1 plasmid, first isolated from mites, is widely found in isolates from cattle, sheep, and goats. Also, in a study by Porten *et al.* **(30)**, nine isolates with plasmid were obtained from sheep and one from a human that was the causative agent of Q fever in North Rhine-Westphalia in 2003 **(30)**. In Lofis *et al.* **(31)**, a sample of chronic form plasmid (QpRS) was isolated from goat milk so that five of the six samples of cow's milk contained acute plasmid (QpH1) **(31)**.

At the same time, in the study of Hilbert *et al.* **(32)** in Germany, all samples exhibited plasmid QpH1; such type of plasmid was the predominant one in isolates in Germany and the Netherlands **(32)**. As a whole, the existence of a species of *C. burnetii* in buffalo and cattle milk of West Azerbaijan province was confirmed in the present research.

All *C. burnetii* positive samples gave identical profiles with the Nine Mile reference strain in the RFLP method. These results confirmed the previously reported by Spitalska **(33)** and Spyridaki, *et al.* **(34)**. PCR-RFLP is a suggested method for the detection and identification of *C. burnetii* from clinical samples originating in humans and animals **(33, 34)**. While the *C. burnetii* genome is still considered highly conserved, according to prior research, *C. burnetii* may be differentiated by RFLP of plasmids **(35, 36)**. The first QpH1 plasmid was described by Loftis et al. **(31)**. It was isolated from *C. burnetii* of the Nine Mile strain. The QpRS plasmid associated with the chronic form has also been described by Samuel *et al.* Chronic form plasmids have been isolated from goat placenta and humans with a chronic form of Q fever. PCR-RFLP is promising for diagnosing *C. burnetii* in the early culture stage, diagnosing acute and chronic forms of infections, and identifying bacteria from specific clinical specimens (heart valves) **(37, 38)**. The profiles of the strains isolated from Q fever patients in France and Greece were reported to be identical with the reference *C. burnetii* Nine Mile strain from ticks originating from the USA **(39)**.

In this study, profiles of all positive samples were also found to be similar to the reference Nine Mile RSA493 strain by RFLP. It also showed that it was impossible to easily conclude the type of plasmid and the course of *C. burnetii* infection. Therefore, the different outcomes of this disease cannot be determined by the type of plasmid. Perhaps other differences between the *C. burnetii* isolates encoded on the chromosomes are likely to play an important role. Recently, analysis of RFLP patterns has revealed significant differences in chromosome size from different *C. burnetii* restriction groups **(40)**.

According to the results of the present study, PCR-RFLP of the amplified plasmid fragment in the positive samples showed no difference in enzymatic digestion patterns between samples. Therefore, there is no genetic heterogeneity among them, and all Coxiellas isolated from milk in the region of West Azerbaijan are the same.

5. Conclusion

The present study concludes no differences in RFLP patterns on *C. burnetii* plasmids. Therefore, there is no genetic heterogeneity among them, and all Coxiellas isolated from milk in the West Azerbaijan province are genetically identical. So, the infection in the different animals can be originated from one strain of *C. burnetii (*Nine Mile RSA493 strain).

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funding the project. Mr. Kazemnia's technical assistance was also highly appreciated.

Conflict of Interest

The authors declared there is no conflict of interest.

Author Contribution

P.K, A.O, K.M, and M.K conceptualized the study. P.K, A.O, K.M collected, analyzed, interpreted the data, and conducted the statistical analysis. P.K, A.O, and K.M wrote the first draft of the manuscript. All authors revised the manuscript and approved the final version. P.K and A.O had full access to all of the data in the study, and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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